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Serologic Investigations of Human Alveolar Hydatid Disease by Western Blotting and Indirect Histo-immunoperoxidase Techniques

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Abstract

The specificity of human antibodies to larval *Echinococcus multilocularis* (Em) was studied by two different techniques of Western blotting (WB) and indirect histo-immunoperoxidase (IP). Forty-two of 49 AHD patients' sera tested by WB showed multiple bands, with especially prominent ones at 55 and 66 kDa. When the 49 AHD patients' sera were tested by IP, about 60% of the specimens revealed strong antibody activity to the germinal layer, brood capsules and protoscoleces of larval Em cysts of the liver of infected Mongolian gerbils. No staining occurred in the laminated layer. All of the IP-positive sera revealed binding activity reactive to the 55 kDa band and the 66 kDa band by WB. Further experiments using antibodies which bound to and were eluted from the 55 and 66 kDa bands confirmed that human anti-55 kDa (or 66 kDa) protein antibody strongly interacted with the germinal layer of larval Em cysts in the liver of infected Mongolian gerbils.

Key words: *Echinococcus multilocularis,* Human alveolar hydatidosis, Western blotting, Indirect histo-immunoperoxidase

Introduction

Alveolar hydatid disease (ADH) is caused by the metacestode of *Echinococcus multilocularis* (Em). The period between infection and clinical manifestation often lags more than 10 years, because the initial symptoms of the disease are usually vague and AHD is often not diagnosed until it is well advanced and inoperable (Kasai *et al.*, 1980). These problems underscore the importance of serologic screening for early detection of AHD. This preventative measure may reduce AHD morbidity and mortality and also offers an opportunity for surgical cure or chemotherapy.

Serologic tests for echinococcosis rank among the most successful immunodiagnostic procedures for the diagnosis of human helminthiases (Williams, 1979). However, until recently, the development of highly sensitive immunoassay systems using enzymes or isotopes has been limited by the lack of purified antigen preparations from the Em metacestode. Some improvements have been reported by Gottstein (1983, 1985), who purified a highly specific antigen from Em and applied it to the immunodiagnosis of serologically or clinically confirmed patients with AHD, using an enzyme-linked immunosorbent assay (ELISA), so called Em2-ELISA (Gottstein *et al.*, 1985; Gottstein *et al.*, 1986). In our laboratory, the Western blotting method was applied to the immunoserologic diagnosis of AHD and was found to be useful for confirming positive results obtained by other tests such as complement fixation test (CFT), indirect haemagglutination (IHA) and immunoelectrophoresis (IE), and for characterizing the specificity of detected antibodies (Furuya *et al.*, 1987).

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However, the specificity of the antibodies contained in the sera of AHD patients has not been fully elucidated, perhaps because of the extreme complexity of antigenicity of the Em metacestode.

The aim of the present study was to clarify the specificity of human antibodies to the Em metacestode. The antibody specificity was demonstrated by Western blotting (WB) and histoimmunoperoxidase (IP).

Materials and Methods

1. Preparations of Em antigen

Protoscoleces-containing organisms were prepared from the Alaskan strain of Em (kindly provided by Prof. R.L. Rausch), which has been maintained in our institute by i.p. passage through the cotton rat (*Sigmodon hispidus*), as described by Norman and Kagan (1961). Briefly, cysts were minced with scissors in Dulbecco's phosphate buffered saline (PBS, pH 7.4), and then were filtered through a 0.6—0.7 mm hole size mesh. The organisms were washed extensively in PBS by gravity sedimentation. To one volume of the gravity-sedimented organisms was added an equal volume of 0.02 M Tris-HCl buffer, pH 7.4, containing 1 M NaCl and 2% Triton X-100 (Tx). This mixture was then disrupted twice by sonication under cooling conditions, using a Kubota sonicator (Tokyo), for a total of 5 min at 20 KHz. The sonicates were centrifuged at 10,000 r.p.m. for 1 hr at 4°C to remove cellular debris, and the supernatants were stored in 100- μl aliquots at —85°C until used.

2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The Em antigen preparation described above was mixed with an equal volume of sample buffer (0.125 M Tris-HCl, pH 6.8, 0.2 M 2-mercaptoethanol, 4% SDS, 40% glycerol, and 0.002% bromophenol blue), and the mixture was heated at 100°C for 3 min. A 300 μl sample (85 μg of protein/single well) was used for electrophoresis and electrophoresed in SDS-PAGE according to Laemmli (1970). Gels contained 8% acrylamide (with 2.6% bisacrylamide). High molecular weight markers (SDS-6H; Sigma Chemical Company, St. Louis, Mo.) were included in each run. Resolved proteins were then transferred to nitrocellulose (NC) membranes (BA 83, Schleicher and Schuell, West Germany) as described by Towbin *et al.* (1979). Narrow vertical strip blotting marker proteins were cut from two sides of the NC sheet and stained with amido black according to Szewczk and Kozloff (1985). The remaining part of the sheet was cut further into strips (2—3 mm wide length) for the detection of anti-larval Em proteins in human sera. The cut strips were blocked with 20% (v/v) fetal calf serum (FCS) and incubated for 1 hr at room temperature. Initial binding by patient serum diluted 1:200 in PBS containing 20% FCS (PBS-FCS) was performed for 2 hrs' incubation at room temperature with gentle shaking. The NC strips were washed with three changes of PBS containing 0.05% Tween 20 (PBS-T) for 15 min at 5 min intervals and subsequently incubated for 1 hr at room temperature with anti-human IgG (γ- chain specific) conjugated with ALP, diluted 1:1,000 in PBS-FCS. After washing, as described above, the strips were incubated with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, which developed a purple color at the bands of antigen-antibody-complex, as described by Learly *et al.* (1983).

3. Elution of specific antibodies to the 55 and 66 kDa proteins of larval Em antigens

Larval Em antigens were fractionated on NC membrane by the WB procedure as described above, and the corresponding areas were cut from the NC membrane. The excised NC strips were incubated with a pool of sera from 3 AHD patients (diluted 1:200 in PBS-FCS) for 1 hr at room temperature and washed extensively in PBS-T. Each NC strip was then washed twice with a small portion of 3 M NaSCN, pH 6.8, and the washes were pooled and dialyzed against PBS at 4°C. The protein concentrations of specific antibodies against 55 kDa protein and 66 kDa protein, which were determined by the Bio Rad protein assay with bovine gamma globulin as

standard, were 61.2 μg/ml and 45.6 μg/ml, respectively. The eluted antibodies bound with new NC strips, conferring electroblotted Em antigens, as shown in Fig. 10.

4. Histo-immunoperoxidase reaction

Em cysts (2—6 mm in diameter) excised from the liver of Mongolian gerbils (*Meriones unguiculatus*) after 2 months of infection were frozen immediately in cold n-hexane in dry ice acetone, sectioned at 5 μm thick and mounted onto microscope slides. After being fixed in 3% formalin (pH 7.4), and 1% Tx and PBS containing 1% FCS (1% FCS-PBS), the tissue sections were exposed to dilute sera (1:500) or eluted antibody preparations and stained by the indirect immunoperoxidase (IP) method described by Pauli *et al.* (1984), except that the first reaction of the dilute sera took 30 min at room temperature. Positive areas were stained reddish brown in color.

Control experiments for eluted antibodies were carried out using normal human IgG at appropriate concentrations.

In addition, some of the slide preparations used in the above experiment were stained to confirm the location of the laminated layer by the periodic acid Schiff (PAS) method (Clark, 1981).

5. Sera tested

The patients were diagnosed as AHD when alveolar hydatid cysts were confirmed surgically, as described previously in detail by Kasai *et al.* (1980). The final diagnosis of AHD was based on histologic examination of the surgical specimens. Sera were collected from the 49 patients thus diagnosed as AHD at the time of operation. Serological examinations showed positive reaction to at least one of the tests, namely ELISA, CFT, IHA and IE, as described previously (Sato *et al.*, 1983).

This study also included sera obtained from 8 patients with cystic hydatidosis (CHD), which were described previously (Sato *et al.*, 1984), and from 50 healthy adults and 20 individuals with other parasitic diseases, of which 19 cases were diagnosed as diphyllobothriasis, and one case as filariasis, and from 15 patients with liver disorders including carcinoma, cirrhosis and non-parasitic cysts.

Results

Human anti-larval Em antibodies demonstrated by Western blotting

It was found that all of the 49 sera of AHD patients tested were WB positive. Forty-two sera showed various bands with molecular weights ranging from 29 to 205 kilodaltons (kDa) (Fig. 1, lanes 1—6), and the remaining 7 sera revealed a few bands with low molecular weights of 30—35 kDa and/or with higher molecular weights (Fig. 1, lanes 7—9). Each serum showing various bands recognized between 10 and 25 components. The most intensively reactive bands had relative molecular weights of 55 (a broad band shown in the region of 53—57 kDa) and 66 kDa (a broad band shown in the region of 60—70 kDa).

Our previous study (Sato *et al.*, 1983) showed that CHD patients' sera reacted with the Em antigen by means of ELISA. To test the cross-reactivity of human anti-Eg antigens with larval Em antigens, 8 sera from CHD patients were examined by the WB method. All of the 8 sera exhibited a week reaction with several bands (Fig. 1, lanes 10—13); however no antibodies against the 55 kDa and 66 kDa proteins were detected, except one serum, of which the WB pattern revealed two clear but thin bands of 55 and 66 kDa (see lane 12 in Fig. 1). This result suggests that the 55 kDa protein or 66 kDa protein are not always species-specific antigen, but they are the most immunoreactive proteins among larval Em antigens. In order to clarify further the specificity of the reaction, 20 sera from patients with other parasitic diseases and 15 sera from patients with liver disorders, as well as 50 samples from healthy adults were tested. None of these sera showed clear bands on the blots carrying larval Em antigens.

Human anti-larval Em antibodies demonstrated by indirect immunoperoxidase

Next, IP reaction of sera from AHD patients

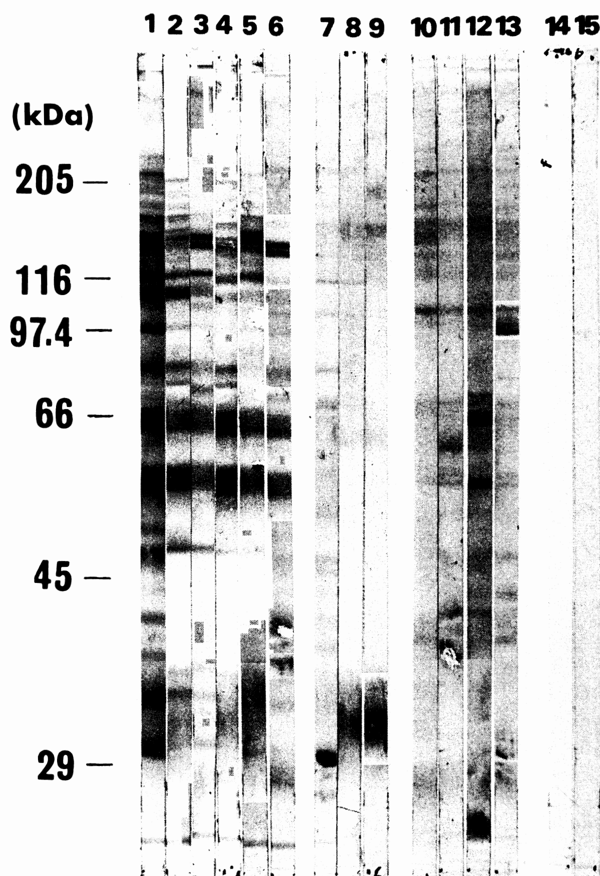
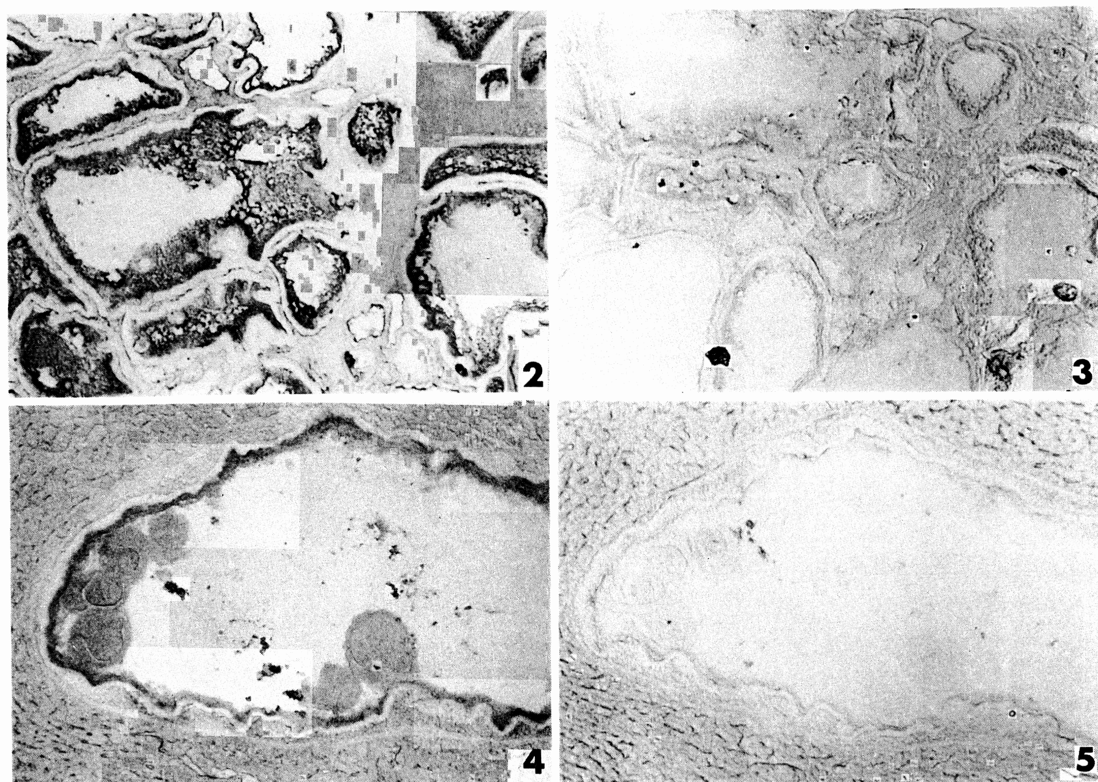


Fig. 1. Representative Western blotting patterns shown by serum samples from patients with hydatidosis
Lanes 1—9: Serum samples from patients with alveolar hydatidosis.
Lanes 10—13: Serum samples from patients with cystic hydatidosis.
Lanes 14 and 15: Serum samples from healthy normal persons.

and CHD patients as well as sera from healthy persons were studied. Thirty-one of 49 AHD patients' sera bound to the germinal layer of the sectioned cysts obtained from Mongolian gerbils (Fig. 2); brood capsules and immature protoscoleces were also IP positive (Fig. 6). In mature protoscoleces, the antigen had a granular appearance in the parenchymal region (Fig. 7), but the reaction of suckers and hooks was judged to be non-specific (Fig. 8), since they reacted with the second antibody of anti-human IgG coupled with peroxidase, but not with the first antibody of tested sera. The laminated layer, which was strongly PAS positive (see Fig. 9), was free from a stainable antigen. On the other hand, only one of 8 sera from CHD patients gave a positive reaction to the germinal layer as well as the brood capsules and the protoscoleces. In contrast to this, none of the 50 control sera from healthy persons reacted with cyst elements, as seen in the bottom row of Table I, which summarizes the results obtained by IP.



Figs. 2—5. Representative indirect immunoperoxidase (IP) staining of the liver lesion of infected Mongolian gerbil using a serum sample from a patient with AHD and a preparation of eluted antibody which bound to the 55 kDa band.

2. IP-positive staining of small vesicles of alveolar hydatid cyst (without protoscoleces) with an AHD patient serum. The antigen is found in the germinal layer.

3. The control section stained with a serum of a normal healthy person. Note the absence of staining of the germinal layer.

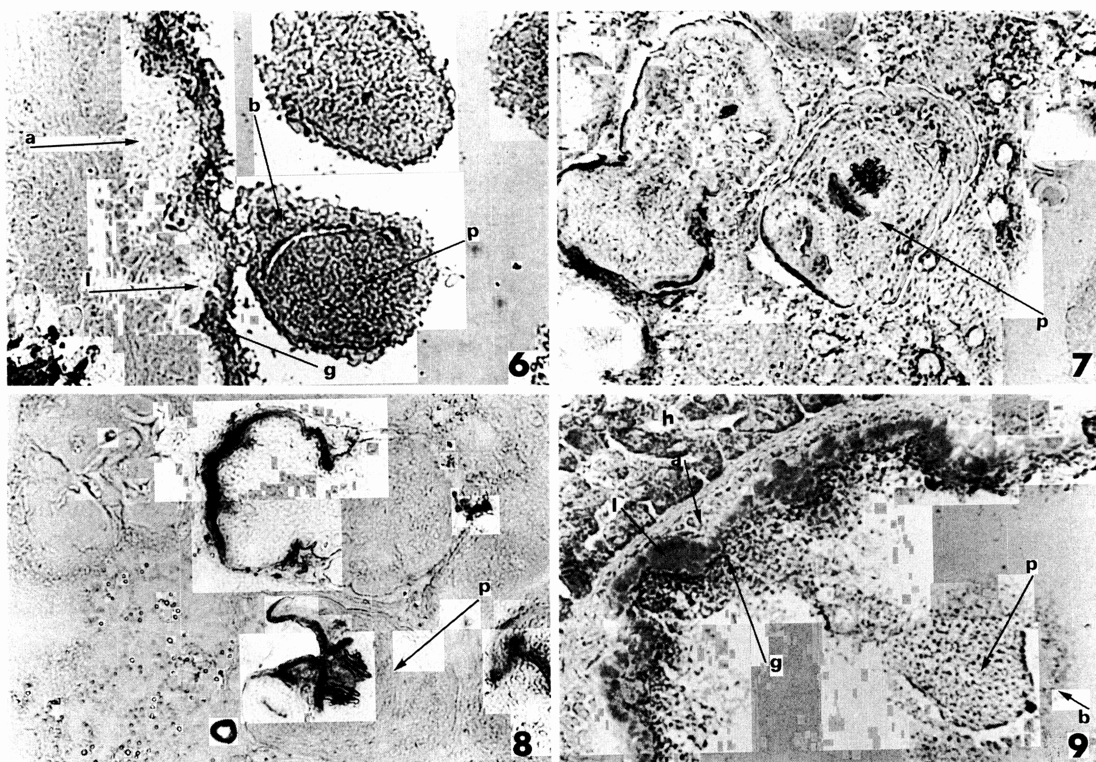
4. The 55 kDa protein-associated antigen is located in the germinal layer, brood capsules and immature protoscoleces, using a preparation of eluted antibody which bound to the 55 kDa band.

5. The control section stained with normal human IgG. Note the absence of staining of the germinal layer, brood capsules and immature protoscoleces.

All figures 40×

IP reactivity of human anti-55 kDa protein and anti-66 kDa protein antibodies

As was stated earlier, the 55 kDa and the 66 kDa bands were the most stainable ones among the larval Em antigens blotted on NC strips, when sera from AHD patients were tested by WB. To demonstrate the location of the 55 kDa protein and the 66 kDa protein in Em cysts, we attempted to elute specific antibodies which bound to the 55 and 66 kDa bands, and found that the eluted antibodies bound to substances contained in the germinal layer, brood capsules and protoscoleces (especially immature ones) in sectioned cysts (Fig. 4). Compared with the reaction of the anti-66 kDa protein antibody (data not shown here), that of the anti-55 kDa protein antibody was considerably clear and strong. However, it is likely that human anti-55 kDa protein cross-reacts with the 66 kDa band, and also that human anti-66 kDa protein reacts with the 55 kDa band, as seen in Fig. 10.



Figs. 6—9.

6. IP-positive staining of the germinal layer, brood capsules and immature protoscoleces using an AHD patient serum. a: Adventitial layer; 1: Laminated layer; g: Germinal layer; b: Brood capsules; p: Immature protoscolex.

7. IP-reactivity of antigen is also found in the parenchyma of mature protoscoleces with an AHD patient serum. p: Mature protoscolex.

8. The control section stained with a serum of a normal healthy person. Note the absence of staining of the parenchyma of protoscolex. However, non-specific staining is observed on hooks and suckers.

9. Periodic acid Schiff (PAS) staining. The laminated layer is demonstrated as strongly PAS-positive material. h: Host tissue; a: Adventitial layer; 1: Laminated layer. g: Germinal layer; b: Brood capsule; p: Immature protoscolex.

All figures 100×.

Table 1 IP reactivity of various structural components of cyst* with sera of patients with either AHD or CHD

Sera tested	Occurrence of specific staining of			
	Germinal layer	Brood capsules	Protoscoleces	Laminated layer
AHD patient sera	31/49	31/49	31/49	0/49
CHD patient sera	1/8	1/8	1/8	0/8
Normal subjects	0/50	0/50	0/50	0/50

*The cysts were obtained from the liver of infected Mongolian gerbils 2 months previously.

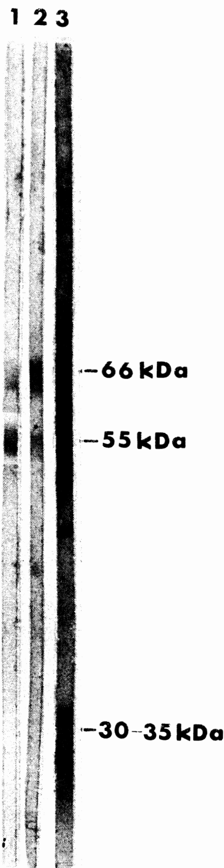


Fig. 10. Western blotting patterns shown by preparations of eluted antibodies which bound to the 55 kDa and 66 kDa bands
Lane 1: A preparation of eluted antibody which bound to the 55 kDa band.
Lane 2: A preparation of eluted antibody which bound to the 66 kDa band.
Lane 3: The reference pattern of staining by the unfractionated AHD serum sample from which anti-55 kDa protein and anti-66 kDa protein antibodies were prepared.

Discussion

A well-known antigen for the serodiagnosis of CHD is the thermolabile, lipoprotein antigen 5 (Capron *et al.* 1967). This antigen is prepared from cyst fluid of Eg and is estimated to have a molecular weight of 69 kDa in SDS-PAGE under reducing conditions (Dottorini and Tassi, 1977). Antigen 5, however, is not suitable for species-specific diagnosis, because antibody to antigen 5 also occurs in the serum of human patients with Em infection (Yarzabal *et al.* 1977). Recently, Gottstein (1983; 1985) developed an ELISA using a purified antigen, called Em2, which is specific to larval Em. This antigen, namely Em2, was shown to be a polypeptide with a relative molecular mass of 54 kDa and an isoelectric point at pH 4.8. The application of the purified polypeptide in ELISA was reported to be 94% sensitive and nearly 100% specific for Em infections using 78 Em patients and 89 patients with other helminthic infections. We assumed that Em2 is identical with the 55 kDa band demonstrated by the present WB method, since the 55 kDa band showed electric mobility similar to Em2 in SDS-PAGE under reducing conditions, and further, was the most immunoreactive one among the electroblotted bands (Fig. 1, lines 1—6).

The 55 kDa band could be detected with the sera of 86% of AHD patients' sera tested by WB. This percentage of sensitivity is lower than that evaluated by Gottstein (1985), as described above. The difference might be attributed to the assay techniques used, i.e. Em2-ELISA by Gottstein (1985) and WB by us. Another possibility is that the cyst state of the patients studied in the present study might be different from that of the patients reported by Gottstein (1985). In this regard, Rausch *et al.* (1985) have recently reported some cases of AHD patients with low or negative activity of antibody in Em2-ELISA. These cases were diagnosed as asymptomatic AHD of the liver. Similar findings were obtained from 7 AHD patients studied in this paper. The 7 cases were found not to interact with 55 kDa band (Fig. 1, lanes 7—9), and they had small lesions that were in the early stage of infection or were dead (data not shown here). Therefore, the state of the cyst is considered to play an important role on immune responses.

The germinal layer of Em cysts could be stained with eluted anti-55 kDa protein antibody as well as AHD sera followed by a peroxidase-labeled anti-human IgG (Figs. 2 and 4). This result means that antibody reactive to the 55 kDa band was elicited by immunological stimulation

against the germinal layer. In fact, 31 of 49 serum samples from AHD patients tested in this study showed antibody activity directed to the germinal layer (Table I). Since germinal cells are the progenitors of proliferating cysts (Sakamoto and Sugimura, 1970; Vogel, 1978; Eckert *et al.*, 1983), the detection of anti-55 kDa protein antibody or anti-germinal layer antibody may indicate the presence of proliferating cysts. However, the significance of this antibody in sero-diagnostic testing requires further study. As described in Table I, 31 AHD patients' sera showing multiple bands with significant prominence at 55 and 66 kDa by WB were IP-positive. However, 11 AHD patients' sera were found to be IP-negative but were 55 (or 66) kDa band-stainable. This is thought to be attributed to the difference in sensitivity between WB and IP. In the former, ALP-coupled anti-human immunoglobulin was used, while in the latter, peroxidase-coupled anti-human immunoglobulin was used. According to Avrameas *et al.* (1978), peroxidase, which is normally very useful in immunohistochemical techniques, might not be suitable in enzyme immunoassay. Our experience with 49 AHD patients' sera suggested that the sensitivity of WB was higher than that of IP. Sensitivity also might be influenced by the conditions of testing such as dilution of serum tested and incubation time for the first antibody. Serum dilution and the first incubation time for WB were 200-fold and 2 hours, while those for IP were 500-fold and 30 min, as shown in the text.

Experiments using AHD patients' sera and antibodies which bound to and were eluted from the 55 kDa and 66 kDa bands indicated that both antigens were derived from the germinal layer, brood capsules and protoscoleces, as mentioned above. This finding supports the view of Kagan and Norman (1961), who pointed out that cross-reaction occurs among those tissues. Thus eluted anti-55 kDa (or 66 kDa) protein antibody could not discriminate among the germinal layer, brood capsules and protoscoleces, because we used cyst legions from Em-infected experimental animals such as Mongolian gerbils and cotton rats as antigen preparations for the IP test. However, it is reasonable to consider that the anti-55 kDa (or 66 kDa) protein antibody contained in AHD patients' sera was produced by immunologic stimulation against the germinal layer alone, as cyst tissues in man rarely contain islands of brood capsules with protoscoleces (Rausch and Wilson, 1973).

Yarzabal *et al.* (1976) found that the laminated membrane of hepatic hydatid cysts, which was recovered from naturally parasitized horses with Eg, did not contain antigenic determinants in common with any of the components of the hydatid fluid, using rabbit anti-whole hydatid fluid serum. Rickard *et al.* (1977) demonstrated that both the anti-antigen 5 antibody and the anti-antigen B antibody bound to the laminated membrane of Em cysts, as well as to the germinal zone. Ali-khan and Siboo (1981) reported that antibodies in CHD patients' sera bound to antigenic determinants of Em, and these determinants were found to be located in both the laminated layer and the germinal layer by the indirect fluorescent technique. Our results showed that the strongly PAS-positive laminated layer did not react with sera from the patients with AHD under our conditions of testing. Thus, it is still an open question whether the laminated layer has an antigenic or immunogenic nature in man.

The experiments of WB suggested that AHD patients may be classified into two serologically different types from their staining pattern: one type, which we tentatively called the "complete type", showed multiple bands with various molecular weights ranging from 29—205 kDa (as seen in Fig. 1, lanes 1—6); another type, called tentatively the "incomplete type", revealed a few bands with low molecular weights of 30—35 kDa and/or with molecular weights higher than 90 kDa (as seen in Fig. 1, lanes 7—9). In a recent study on the duration of antibody persistence after operation, we demonstrated that patients classified into the "incomplete type" stopped producing antibodies against 30—35 kDa proteins within one year, while patients classified as "complete type" maintained a constant level of antibody production to the cystic antigens for over one year (manuscript in preparation). Further research on the serodiagnostic significance of the two types, based on WB patterns,

is now under way in our laboratory.

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